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Evaluation and Analysis of Growth, Maximum Bioaccumulating Properties of *Penicillium chrysogenum* MTCC 6477 on Reactive Black 5 Dye

M. Anil Kumar[1], S. Keerthana[2], Adyasa Pani[3], D. Suresh[4], C. B. Lakshmikantha[5], M. Seenuvasan[6]

1-4,6 Department of Biotechnology, Madha Engineering College, Chennai, INDIA
5 Principal, P.B.College of Engineering, Chennai, INDIA

seenuchem786@gmail.com, lakshmikantha.cb@rediffmail.com

ABSTRACT:
The growth and bio accumulating property of *Penicillium chrysogenum* MTCC 6477 on Reactive Black 5 (RB5) dye was tested under molasses sucrose and dye concentrations. The highest specific growth rate (μ) of 0.115 h^{-1} was obtained at an initial sucrose concentration of 15 g/L in the absence of dye. For each constant sucrose concentration (5 to 15 g/L), the initial dye concentration was varied (0 to 500 mg/L) for each trial; this resulted in the decrease in bioaccumulation percent yield and increase in maximum bioaccumulation capacity (q_m). But, all dye concentrations tested was found to inhibit the mycelial growth and the non-competitive inhibition model can express this. On the other hand, at each constant dye concentration the μ and percent yield was enhanced when sucrose concentration was raised and q_m was found to be decreasing in a slower manner. The maximum yield of 99.4% was observed in the growth medium containing 15 g/L sucrose and 50 mg/L dye. The effects of initial sucrose and dye concentrations on μ and percent yield of mycelia were analyzed by statistical method and two model equations and the responses were developed by Response Surface Methodology (RSM).

Key Words: Bioaccumulation, RB5 dye, *Penicillium chrysogenum*, Specific growth rate, percent yield, RSM

INTRODUCTION

Highly coloured synthetic dye effluents from textile, food, paper and cosmetic industries are been released into the receiving water and these contaminate the water resources [1]. The residual colour is a problem with reactive dyes because; in current dyeing process nearly 50% of the dye is lost into the wastewater [2]. It is known that the very small amount of dye in water (i.e.10-50 mg/L) affects the aesthetic value, water transparency and gas solubility of water bodies. Usually the dye wastewater is treated by physical- or chemical- treatment processes. These include chemical coagulation/flocculation, ozonation, oxidation, ion exchange, irradiation, precipitation and adsorption [3-7]. Some of these techniques have been shown to be effective, although they have limitations [8, 9]. In recent years, a number of studies are focused on some microorganisms that are able to biodegrade or bioaccumulate azo dyes in wastewaters [3, 4, 10, 11]. Dead/pre-treated biomass is also used for sorption of dye and many other pollutants (i.e. biosorption). The non-viable biomaterials and isolated potent microorganisms are considered to be limited for real application due to economics, non-availability, low binding capacity, efficiency and system control difficulty [12-17]. The other biological treatment method; bioaccumulation is defined as the accumulation of pollutants by actively growing cells. In metal bioaccumulative process, there is an initial rapid accumulation step, i.e., metabolism- and temperature- independent, and this is thought to involve ion binding to the cell wall structure. The second process follows this step, i.e., metabolism-dependent, which is slower but can accumulate larger quantities of ions than that of the first process. Decolourisation of the dye solution could also be due to adsorption of the dye on the biomass or bioaccumulation. Using growing cultures in removal process will avoid the need for a separate biomass production process (e.g. cultivation, harvesting, drying, processing and storage prior to use). There are significant practical limitations in the biouptake process, which employs living cell systems. Perhaps the most significant limitation is the inhibition of cell growth when there is too high dye concentration. Active uptake processes also require metabolic energy, which should be externally provided by means of easily usable carbon and nitrogen sources instead of dye in the growth media. If the problem of dye toxicity to the growing cells is overcome by the use of dye resistant organisms [17-21]. The classical method of studying with one variable at a time can be effective in some cases but it is not capable of representing the combined effects of all the factors involved. The response surface methodology (RSM) can be employed as an investigator strategy to implement process conditions which drive to optimal response by performing a minimum number of experiments. Recently several studies have described the use of RSM for optimization of process parameters, such as pH, pollutant concentration, and biosorbent dose for biosorption of metals or dyes from synthetic solutions [22-25].

The present work evaluates the use of cane molasses as an alternative cheaper growth enriched media due to its

high sucrose and other nutrient contents, on the growth and bioaccumulating properties of *Penicillium chrysogenum* in the batch scale.

MATERIALS AND METHODS

Dye Solution

RB5 dye is a commonly used textile dye was purchased in a pure form. The dye stock solution was prepared by dissolving the powdered dyestuff in distilled water to a final concentration of 1000 mg/L and was stored in the dark at room temperature.

Microorganism and Growth Conditions

The microorganism Penicillium *chrysogenum* MTCC 6477 used for this study was obtained from MTCC (Microbial Test Culture Collection and Gene Bank, Chandigarh) and was maintained in the Czapek's Yeast Extract Agar medium (Sucrose 30.0 g, yeast extract 5.0 g, agar 15.0 g, K_2HPO_4 1.0 g, $NaNO_3$ 30.0 g, KCl 5.0 g, $MgSO_4$. $7H_2O$ 5.0 g, $FeSO_4.7H_2O$ 0.1 g in a litre of distilled water and were subcultured for every 30 days. It was grown in the above said medium constituents in the absence of agar. To check the decolourisation ability of the microorganism some trail experiments were performed in the presence and absence of carbon and nitrogen sources in the Czapek's medium along with the Reactive Red C2G 29 dye. In order to produce more resistant and efficient strain, adaptation of the cells to progressively higher concentrations of dye was performed. Microbial adaptation is defined as the ability of a microbial population to adjust itself to a changing environment [26]. Adapted strain was obtained during serial subcultures in growth medium supplemented with different concentrations of RB5 dye changing between 50 and 500 mg/L at a constant sucrose concentration varied for each experimental set. The culture grown in the medium containing RB5 dye at the lowest level was transferred to the next medium supplemented with a higher concentration of dye and thus, acclimatized to higher concentrations of dye at the same sucrose concentration. Acclimation usually involves the use of alternative metabolic pathways, which are not disrupted (or least disrupted to a lesser degree) by the presence of dye anions. When the adapted culture reached to its exponential growth phase, 1.5 mL of the culture medium was transferred to the next culture medium. The adaptation studies were repeated two times for each RB5 dye and sucrose concentration.

Dye Bioaccumulation Assays

In order to prepare the bioaccumulation medium, RB5 dye solution was autoclaved separately, and then it was mixed with the sterilized growth medium including molasses sucrose in varying concentrations from 5 to 15 g/L and 1.0 g/L $(NH_4)_2SO_4$ and 1.0 g/L KH_2PO_4. An aliquot (1%, v/v) of an adapted pre-culture harvested from exponential growth phase was transferred to fresh media (50 mL) supplemented with RB5 dye by varying the concentration from 50 to 500 mg/L at a constant sucrose concentration. The cultivation was carried out at 37 ˚C in incubator up to its saturation time.

The dye bioaccumulation properties of the mycelia were investigated in a batch system as a function of RB5 dye concentration. The results are given as C_{acc}, X and q_m.

Based on the mass balance principle:

$$q_m = \frac{C_{acc}}{X} \qquad (1)$$

Bioaccumulation percent yield is defined as the ratio between bioaccumulated concentrations of dye, C_{acc} at the end of microbial growth to the initial dye concentration, C_0.

$$Percent\ yield = \left(\frac{C_{acc}}{C_0}\right) \times 100 \qquad (2)$$

Application of Monod Model

The relationship between the rate-limiting substrate (sucrose) concentration and specific growth rate in the absence of inhibitory substance often assumes the form of saturation kinetics and can be described by the Monod equation [27]:

$$\mu = \frac{\mu_m}{1+(K_S/S)} \qquad (3)$$

When sucrose is used as the main substrate in the medium in the presence of higher concentrations of inhibitory substances such as dyes, microbial growth becomes inhibited, and growth rate depends on inhibitor concentration.

Table 1: Experimental ranges and levels of the independent variables.

Independent variables	Design variables	Range and Levels		
		-1	0	1
S_0 (g/L)	X_1	5	10	15
C_0 (mg/L)	X_2	50	275	500

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Table 2: The full factorial central composite design matrix of two variables in coded and uncoded values.

Run	X_1	X_2	S_0 (g/L)	C_0 (mg/L)
1	-1	-1	5	50
2	1	-1	15	50
3	-1	1	5	500
4	1	1	15	500
5	-1	0	5	275
6	1	0	15	275
7	0	-1	10	50
8	0	1	10	500
9	0	0	10	275
10	0	0	10	275
11	0	0	10	275
12	0	0	10	275
13	0	0	10	275

Table 3: Comparison of specific growth rate, percent yield and maximum bioaccumulating capacity at varying levels of sucrose and RB5 dye concentration.

S_0 (g/L)	C_0 (mg/L)	μ (h^{-1})	*Percent yield* (%)	q_m (mg/g)
5	0	0.080	0	0
10	0	0.106	0	0
15	0	0.115	0	0
5	50	0.074	84	1.253
10	50	0.097	96	1.048
15	50	0.107	99.4	0.974
5	100	0.069	77.5	2.672
10	100	0.090	85	2.12
15	100	0.100	93	1.867
5	275	0.056	74.54	9.111
10	275	0.073	83.61	8.579
15	275	0.081	90.11	8.26
5	500	0.045	69.6	19.88
10	500	0.059	80	19.047
15	500	0.065	87	18.125

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Table 4: Comparison of the values of specific growth rate and percent yield obtained experimentally and predicted from RSM.

Run	S_0 (g/L)	C_0 (mg/L)	μ (h^{-1}) $_{exp}$	μ (h^{-1}) $_{pred}$	*Percent yield(%)* $_{exp}$	*Percent yield(%)*$_{pred}$
1	5	50	0.074	0.075	84	84.867
2	15	50	0.107	0.108	99.4	99.990
3	5	500	0.045	0.045	69.6	69.600
4	15	500	0.065	0.065	87	86.724
5	5	275	0.056	0.055	74.54	73.672
6	15	275	0.081	0.082	90.11	89.795
7	10	50	0.097	0.096	96	94.542
8	10	500	0.059	0.059	80	80.275
9	10	275	0.073	0.073	83.61	83.846
10	10	275	0.073	0.073	83.61	83.846
11	10	275	0.073	0.073	83.61	83.846
12	10	275	0.073	0.073	83.61	83.846
13	10	275	0.073	0.073	83.61	83.846

Table 5: Analysis of variance (ANOVA) for quadratic model for specific growth rate.

Sources of variation	Degrees of Freedom	Sum of squares	Mean Square	F-value	P
Regression	5	0.003	0.00063	1672.29	0
Residual Error	7	3.0 E-06	0		
Lack-of-Fit	3	3.0 E-06	1.0 E-06		
Pure Error	4	0	0	0	
Total	12	0.003			

R^2=0.999

Table 6: Analysis of variance (ANOVA) for quadratic model for bioaccumulation percent yield.

Sources of variation	Degrees of Freedom	Sum of squares	Mean Square	F-value	P
Regression	5	733.14	146.628	227.51	0
Residual Error	7	4.511	0.644		
Lack-of-Fit	3	4.511	1.504		
Pure Error	4	0	0	0	
Total	12	737.651			

R^2=0.994

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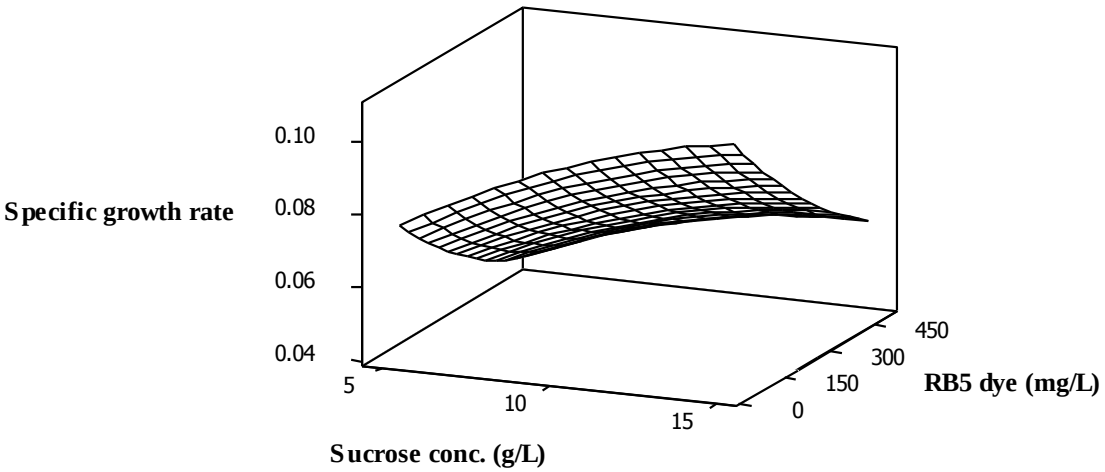

Fig. 1. Surface plot of Growth rate vs. Sucrose concentration, Dye concentration.

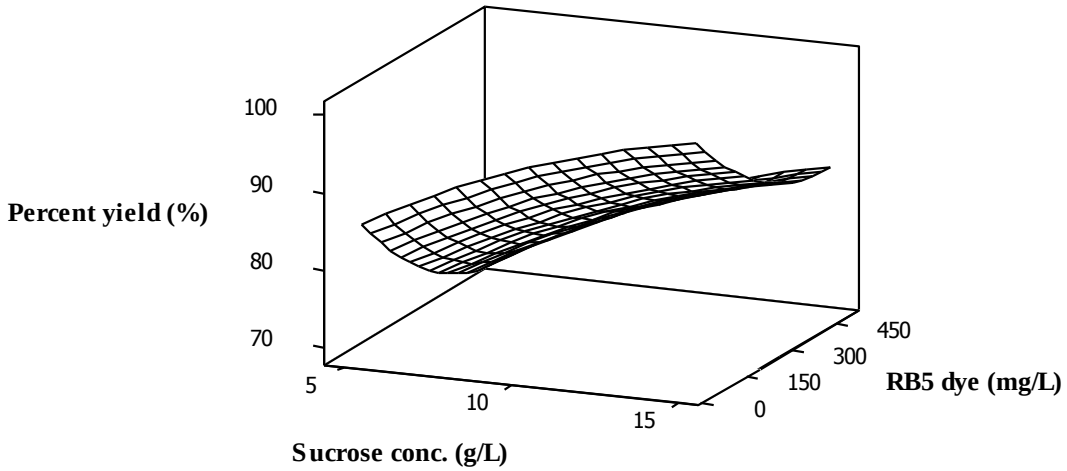

Fig. 2. Surface plot of percent yield vs. Sucrose concentration, Dye concentration.

The following non-competitive inhibition model describing dye component inhibition was selected for assessing the dynamic behavior of mycelia.

$$\mu = \frac{\mu_m}{(1+K_S/S)(1+C_o/K_I)} \quad (4)$$

The value of inhibition constant of dye anions, K_I could also be estimated by non-linear regression techniques using the data on the specific growth rate obtained at different initial dye concentrations, at a constant sucrose concentration changed for each experimental set assuming $S = S_0$ at the beginning of the exponential growth.

Analytical Methods

During the incubation period, a 4 mL sample was taken at fixed time intervals from each flask for the analysis of microorganism and residual RB5 dye concentration in the culture media. The samples were centrifuged at 12,000 rpm for 10 min to precipitate the suspended biomass. The concentration of RB5 dye in the supernatant fraction was analyzed at λ_{max} of 597 nm, absorbance measurements were done by using a UV-VIS Spectrophotometer (ELICO-SL156). Dye free molasses medium was used as blank. The centrifuged cells were washed and resuspended in distilled water. Then the suspension was made up to 4 mL. All the experiments were carried out at least two times. The values used in calculations were mostly the arithmetic average of the experimental data. RB5 dye uptake values were determined as the difference between the initial dye concentration and that of the supernatant.

Two control Erlenmeyer flasks were prepared. First control medium contained molasses without any dye to examine the growth of the mycelia. Second control medium contained both dye and molasses without inoculums to observe any reactions of the media with the dye.

Statistical Analysis

The central composite design (CCD) under the response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables [28]. In this analysis, initial sucrose and RB5 dye concentration chosen as independent variables and the specific growth rate and percent yield as dependent output response variables as shown in Table 1. In order to study the combined effects of these variables on the responses, 13 sets of experiments with appropriate combinations of sucrose concentration and RB5 dye concentration were conducted using statistical method. The first independent variable was varied over 2 levels (5 and 15 g/L) relative to the centre point (10 g/L), the second independent variable was varied over two levels (50 and 500 mg/L) relative to the centre point (275 mg/L). The full factorial central composite design matrixes of two variables with respect to their uncoded and coded values were listed in Table 2. The numerical and graphical analysis for the responses of specific growth and percent yield was estimated using the software MINITAB 14.

Evaluation of the goodness of fit of the model is done through coefficient determination and analysis of variances. The experimental results were fitted to a second order polynomial equation;

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB \quad (5)$$

where, Y is the dependent variable (specific growth rate and percent yield); A and B are the independent variable; β_0 is the regression coefficient at center point; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients and β_{12} is the second order interaction coefficient. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p- and F-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The statistical software package was used to identify the experimental design as well as to generate a regression model to predict the optimum combinations considering the effects of linear, quadratic and interaction on specific growth rate and percent yield. A final experiment was conducted to validate the CCD model developed [22-25, 29-31].

RESULTS AND DISCUSSION

Effect of Initial Sucrose Concentration

There are various factors affecting the growth of cells in a bioaccumulation process such as pH, media composition, dye concentration, etc. The knowledge of these affecting parameters should be investigated. On account of this, the effect of initial sucrose and initial RB5 dye concentration on the mycelial growth and RB5 dye accumulation was studied. The growth was observed that an increasing sucrose concentration is from 5 to 15 g/L in the absence and presence of RB5 dye. The RB5 dye concentration was kept constant between 0 and 500 mg/L.

The μ value increases from 0.08 to 0.115 h^{-1} at an increasing sucrose concentration from 5 to 15 g/L in the absence of dye anions.

Upon the addition of RB5 dye the μ diminished due to the increase in lag and log periods resulting in low bioaccumulation rate (Table 3). This reveals that RB5 dye anion bioaccumulation was dependent on the metabolic activity of the cells. The increase in specific growth rate with the increase in the initial sucrose concentration could be due to cell defence mechanism such as acclimation to toxicity.

Effect of Initial RB5 Dye Concentration

In this study, initially the sucrose concentration was kept constant at 5, 10 and 15 g/L then the RB5 dye concentration was varied from 0, 50, 100, 275 and 500 mg/L for. The Table 3 reveals that the maximum specific growth rate occurs in absence of RB5 dye. The dye anion in the molasses medium inhibits the growth rate irreversibly. This inhibition of growth rate occurs with increase of RB5 dye concentration for all concentration of sucrose. In 5 g/L of sucrose the specific growth rate was ceasing from 0.08 to 0.045 h^{-1} for the rise in RB5 dye concentration from 0 to 500 mg/L i.e., they showed about 43.125% of reduction. When sucrose concentration was increased to 15 g/L the mycelial growth rate decreases from 0.115 to 0.065 h^{-1}, for the change in RB5 dye concentration there is about 42.69% reduction. It was evident that sucrose concentration has a pervasive role in mycelial growth rate and decreases the inhibitory effects of RB5 dye on the specific growth rate.

Non competitive Inhibition Model

The values of μ_m and K_S were found to be 0.149 h^{-1} and 4.323 g/L using the double reciprocal form of Monod equation in the absence of RB5 dye. The presence of increasing dye concentrations inhibited the mycelial specific growth rate that indicates the inhibition by RB5 dye following the non-competitive inhibition model. Using the (Eqn. 2) the value of the average inhibition constant, K_I was found to be 656.412 mg/L. The value of the kinetic constant confirms the inhibition effect RB5 dye on the growth of *P. chrysogenum*.

Bioaccumulation of RB5 Dye by Penicillium chrysogenum

RB5 dye bioaccumulation is largely affected by the initial RB5 dye and sucrose concentration. The bio accumulating properties such as percent yield and q_m is listed (Table 3). It shows that change in sucrose and RB5 dye concentrations had a large impact on dye bioaccumulation and yield. For instance at a constant dye concentration of 50 mg/L with various sucrose concentrations from 5 to 15 g/L The q_m value found to decrease from 1.253 to 0.974 mg/g and yield was found to increase from 84 to 99.4%. Similarly at a constant dye concentration of 500 mg/L with varying sucrose concentration from 5 to 15 g/L the q_m decreased from 19.88 to 18.125 mg/g and *Y%* increased from 69.6 to 87% which infers that the raise in the sucrose concentration shows the least effect on q_m and a significant effect on percent yield. Describing, that the mycelial cells requires more nutrients and q_m mainly depends on increasing concentration of RB5 dye. Hence, for a bioremoval process appropriate sucrose concentration should be maintained for maximal dye uptake.

The dye accumulation is dependent on both sucrose and dye concentration. The raise in the inhibition affect growth and this is due to the raise in dye concentration. The value of q_m increases with increase in the RB5 dye concentration but the yield decreases (Table 3). At 5 g/L of sucrose and 50 mg/L of dye, the q_m is 1.253 mg/g and the yield is 84% but at the same sucrose concentration and 500 mg/L of dye, the q_m is 19.88 mg/g and the *Y%* is 69.6%. This infers that the initial RB5 dye concentration had a significant effect on both q_m and percent yield this is due to an important driving force provided by the initial concentration to overcome all mass transfer resistances of the dye between the aqueous and solid cell phases.

Response Surface Estimation

The need to ensure the effectiveness of biological waste water treatment using the growing cells has stimulated the use of mathematical models for predicting microbial behavior. The objective of this study is to investigate the combined effects of initial sucrose concentration and RB5 dye concentration (independent variables) and was optimized for the maximum growth and percent yield. Experiments were carried out as per the design matrix of the central composite design (CCD), and the average obtained from the culture was used as response. The experimental and the predicted values agreed very well when analyzed in MINITAB 14 (Table 4). ANOVA was carried out for the results of quadratic models for the specific growth rate and percent yield as shown in the Table 5 and 6. The associated probability > F value for each model is lower than 0.05. At the model level, the correlation measure for the estimation of the regression equation is the determination coefficient R^2. The correlation between the experimental and predicted values is better when the value of R^2 is closer to 1.0. In this experiment, the value of R^2 for μ and percent yield were 0.999 and 0.994, respectively. These values indicate a high degree of correlation between the experimental and the predicted values. The value of R^2 indicates that 99.9% and 99.4% of the variables: initial sucrose concentration and RB5 dye concentration contribute very positively to the responses. The value of R^2 is also a measure of fit of the model and it can be mentioned that only about 0.1% of the total variations were not explained by the specific growth rate and 0.6% of the total variations were not explained by the percent yield Linear and quadratic effects of parameters were significant, meaning that they can act as limiting nutrient and little variation in their concentration would alter either the growth rate or the product formation rate or both to a considerable extent [32-33].

The 3D response surface plots (Fig.1-2) are the graphical representation of the regression equation used to determine the optimum values of the variables

within the ranges considered. The main target of response surface is to hunt efficiently for the optimum values of the variables such that the response is maximized [35]. An elliptical response surface in the entire region was found from the second order quadratic equation for the specific growth rate and percent yield with the interaction of the independent variables. Further these three dimensional plots are easier and convenient means for optimizing the variables.

CONCLUSION

As a result it can be said that RSM was used successfully as a fast and error free approach for the optimization of parameters for bioaccumulation with respect to the parameters of molasses sucrose as medium component and RB5 dye concentration. Besides, the interaction study between these components provided an additional advantage of employing RSM. By the three-dimensional response surface plots, it is convenient to estimate the optimum levels of two independent variables. The optimal value of specific growth rate and bioaccumulation percent yield was found to be at 50 mg/L RB5 dye and 15 g/L sucrose, the maximum bioaccumulation capacity was found to be always increases with increase in RB5 dye concentration but the organism reaches the death/decline phase in a rapid rate (data not shown).

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